

REVIEW

Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion

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ABSTRACT

Growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) belong to the same superfamily of regulatory neuropeptides and have both been characterized on the basis of their hypophysiotropic activities. This review describes the molecular evolution of the GHRH/PACAP gene family from urochordates to mammals and presents the hypothesis that the respective roles of GHRH and PACAP in the control of GH secretion are totally inverted in phylogenetically distant groups of vertebrates. In mammals, GHRH and PACAP originate from distinct precursors whereas, in all submammalian taxa investigated so far, including birds, amphibians and fish, a single precursor encompasses a GHRH-like peptide and PACAP. In mammals, GHRH-containing neurons are confined to the infundibular

and dorsomedial nuclei of the hypothalamus while PACAP-producing neurons are widely distributed in hypothalamic and extrahypothalamic areas. In fish, both GHRH- and PACAP-immunoreactive neurons are restricted to the diencephalon and directly innervate the adenohypophysis. In mammals and birds, GHRH plays a predominant role in the control of GH secretion. In amphibians, both GHRH and PACAP are potent stimulators of GH release. In fish, PACAP strongly activates GH release whereas GHRH has little or no effect on GH secretion. The GHRH/PACAP family of peptides thus provides unique model in which to investigate the structural and functional facets of evolution.

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INTRODUCTION

Growth hormone-releasing hormone (GHRH) was initially characterized from a human pancreatic tumor on the basis of its ability to stimulate growth hormone (GH) secretion from adenohypophyseal cells (Guillemin *et al.* 1982, Rivier *et al.* 1982).

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first identified from the ovine hypothalamus as a result of its capability of stimulating cAMP formation in rat anterior pituitary cells (Miya *et al.* 1989). Soon after the discovery of PACAP, it became apparent that these two hypophysiotropic neuropeptides exhibit a

	1	5	10	15	20	25	30	35	40	45
PACAP38	H	S	D	G	I	P	T	D	S	R
PACAP 27	-	-	-	-	-	-	-	-	-	a
VIP	-	-	-	A	V	-	-	N	-	a
GHRH	Y	A	-	-	-	N	-	-	K	L
Secretin	-	-	-	T	-	S	E	L	-	E
Glucagon	-	-	-	Q	-	T	-	S	D	-
GLP-2	-	-	-	Q	-	T	-	S	D	-
PPR	D	V	A	H	-	L	N	E	A	-
PHM	-	A	-	V	-	S	D	F	-	K
GIP	Y	A	E	-	T	-	I	S	-	D

FIGURE 1. Primary structures of the different members of the GHRH superfamily in human. (1) Kimura *et al.* 1990, (2) Ohkubo *et al.* 1992, (3) Itoh *et al.* 1983, (4) Rivier *et al.* 1982, (5) Guillenem *et al.* 1982, (6) Ling *et al.* 1984, (7) Carlequist *et al.* 1985, (8) Bell *et al.* 1983, (9) Moody *et al.* 1984. Abbreviations as in the text; a, amidated; -, amino acid identical to that of PACAP38.

FIGURE 2. Comparison of the primary structures of GHRH and PACAP in different species of vertebrates and urochordates. (1) Rivier et al. 1982, (2) Guillemin et al. 1982, (3) Ling et al. 1984, (4) Böhnen et al. 1983, (5) Esch et al. 1983, (6) Brazeau et al. 1984, (7) Ono et al. 1994, (8) Spiess et al. 1983, (9) Böhnen et al. 1984, (10) Frohman et al. 1989, (11) Sühr et al. 1989, (12) McRory et al. 1997, (13) Alexandria et al. 2000, (14) Vaughan et al. 1992, (15) Parker et al. 1993, (16) McRory et al. 1995, (17) McRory & Sherwood 1997, (18) Miyata et al. 1989, (19) Kimura et al. 1990, (20) Miyata et al. 1990, (21) Ogi et al. 1990, (22) Hosoya et al. 1992, (23) Ohkubo et al. 1992, (24) Okazaki et al. 1995, (25) Yamamoto et al. 1998, (26) Pohl & Wank 1998, (27) Charrel et al. 1991, (28) Matsuda et al. 1997a, (29) Matsuda et al. 1998 a, amidated; -, amino acid insertion or deletion of the human GHRH or mammalian PACAP.

number of structural and functional similarities. (i) They stand among the largest hypothalamic neurohormones discovered so far. (ii) They possess considerable sequence similarity so that it is generally accepted that they arose by genomic duplication of the exon encoding a common ancestor. (iii) They both appeared very early during

evolution prior to the emergence of vertebrates. (iv) They are widely distributed in peripheral organs, notably in the gut, pancreas, ovary and testis. (v) Their biological effects are mediated through 7-transmembrane domain receptors positively coupled to adenylyl cyclase. Besides these similarities, GHRH and PACAP display one major

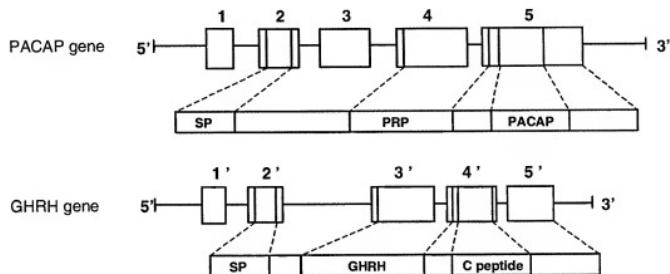
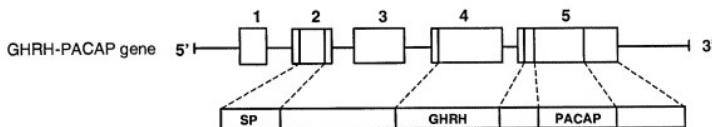
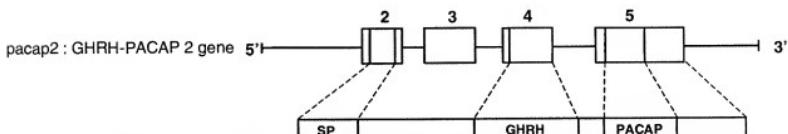
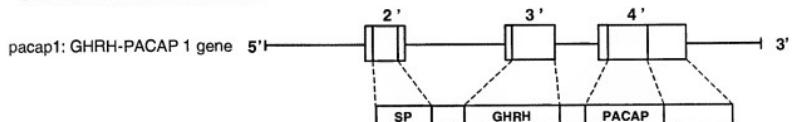
Mammals**Infra-mammalian Vertebrates****Urochordate Invertebrates**

FIGURE 3. Organization of the GHRH, PACAP and GHRH/PACAP genes of vertebrates and invertebrates. Exon numbering follows that used for the mammalian genes.

difference: the sequence of GHRH has been poorly conserved, even in closely related species, while the sequence of PACAP has been extremely well preserved during evolution.

Examination of the amino acid sequences of GHRH and PACAP clearly indicates that these two neuropeptides belong to a superfamily of regulatory peptides which encompasses glucagon, secretin, vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM) or peptide histidine isoleucine (PHI), gastric inhibitory peptide (GIP), and glucagon-like peptides (GLPs) (Fig. 1) (Campbell & Scanes 1992, Kieffer & Habener 1999). Molecular characterization of the GHRH and PACAP precursors in several representative species of urochordates and vertebrates, together with studies on their physiological implication in the control of pituitary hormone secretion, have provided important information pertaining to the evolutionary aspects of their structures and functions. The aim of the present review is to summarize the current knowledge regarding the phylogenetic origin and the evolution of the respective roles of GHRH and PACAP in the regulation of GH secretion.

MOLECULAR DIVERSITY OF GHRH AND PACAP

Evolution of the primary structures of GHRH and PACAP

The sequence of GHRH was initially determined from a tumor which had caused acromegaly (Guillemin *et al.* 1982, Rivier *et al.* 1982). Subsequently, the primary structure of the peptide has been elucidated in a number of species, either directly determined after peptide purification or deduced from a cloned cDNA. In contrast to most hypophysiotropic neurohormones whose sequences have been generally well conserved during evolution, the primary structure of GHRH is highly variable (Fig. 2). In particular, rat and mouse GHRHs only exhibit 67% sequence identity, which is by far the lowest degree of similarity for regulatory neuropeptides observed between these two rodent species.

Two molecular forms of PACAP with 38 (PACAP38) and 27 (PACAP27) amino acids were originally isolated from the sheep hypothalamus (Miyata *et al.* 1989, 1990). The sequence of PACAP has been remarkably well preserved from the sea squirt (an urochordate invertebrate) to the human (Fig. 2). In particular, the sequence of PACAP38 is identical in all mammalian species studied so far. In

other vertebrates, strong evolutionary pressure has acted to preserve the sequence of the N-terminal domain which corresponds to the biologically active region of the molecule (Gonzalez *et al.* 1998). It is noteworthy that the sequences of the two tunicate PACAP27 variants exhibit only one and four amino acid substitutions as compared with their mammalian ortholog (Miyata *et al.* 1990, McRory & Sherwood 1997). Such a high degree of peptide sequence conservation between two taxa which have diverged from a common ancestor some 600 million years ago (McRory & Sherwood 1997) indicates that PACAP fulfills important biological functions.

Evolution of the structure of the GHRH and PACAP precursors

In mammals, the GHRH and PACAP precursors are encoded by two distinct genes (Mayo *et al.* 1985a, Hosoya *et al.* 1992). Each of these precursors encompasses two related peptides: the GHRH precursor comprises GHRH and a C-terminal flanking peptide called C-peptide, while the PACAP precursor comprises PACAP and an N-terminal flanking peptide called PACAP-related peptide or PRP (Fig. 3). Comparison of the sequences of these peptides reveals a higher degree of similarity between PRP and GHRH than between PRP and PACAP (45% and 21% respectively in human).

In contrast, in all submammalian species investigated so far, a GHRH-like peptide and PACAP are located on the same precursor (Fig. 3). To date, the cDNA encoding the GHRH/PACAP precursor has been characterized in the chicken *Gallus domesticus* (McRory *et al.* 1997), the frog *Rana ridibunda* (Alexandre *et al.* 2000), the catfish *Clarias macrocephalus* (McRory *et al.* 1995) and the salmon *Oncorhynchus nerka* (Parker *et al.* 1993). In birds, amphibians and fish, alternative splicing of the primary transcript can generate either a long mRNA encompassing both the GHRH and PACAP sequences, or a short mRNA lacking the GHRH sequence (McRory *et al.* 1997, Parker *et al.* 1997, Alexandre *et al.* 2000). Two cDNAs encoding GHRH/PACAP precursors have also been cloned in an urochordate, the sea squirt *Ciona intestinalis* (McRory & Sherwood 1997). The organization of the GHRH/PACAP genes in urochordates exhibits strong similarities with those of vertebrates (Fig. 3). Sequence analysis of the two GHRH/PACAP precursors of the sea squirt reveals a higher degree of similarity between the two GHRH sequences (89%) and the two PACAP sequences (89%) than between the GHRH and PACAP sequences within each precursor (41% for the GHRH/PACAP1 precursor and 48% for the

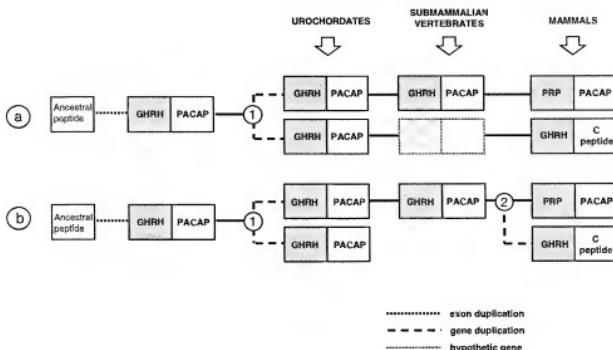


FIGURE 4. Proposed evolutionary history of the GHRH/PACAP precursor family. In both hypotheses a and b, it is assumed that exon duplication, leading to the appearance of the GHRH/C peptide and PRP/PACAP sequences, occurred before gene duplication. Hypothesis a: a single duplication would have occurred before the emergence of urochordates. Hypothesis b: two duplications would have occurred independently in the urochordates and in a common ancestor of mammals.

GHRH/PACAP2 precursor), suggesting that exon duplication generating GHRHII and PACAP occurred before duplication of the ancestral gene (Fig. 4).

Since only one GHRH/PACAP cDNA has been characterized to date in teleosts (Parker *et al.* 1993, McRory *et al.* 1995), amphibians (Alexandre *et al.* 2000) and birds (McRory *et al.* 1997) while two distinct cDNAs encoding either GHRH or PACAP have been cloned in rat (Mayo *et al.* 1985b, Ogi *et al.* 1990), mouse (Frohman *et al.* 1989, Suhr *et al.* 1989, Okazaki *et al.* 1995) and human (Gübler *et al.* 1983, Mayo *et al.* 1983, 1985a, Kimura *et al.* 1990, Hosoya *et al.* 1992, Ohkubo *et al.* 1992), it has been proposed that a gene duplication event occurred just before the emergence of mammals (Arimura 1998, Hoyle 1998, Vaudry *et al.* 2000). However, several observations suggest that a second gene encoding a GHRH-like peptide may well exist in non-mammalian species (Fig. 4). In particular, two GHRH/PACAP genes are present in urochordates, suggesting that duplication of the ancestral gene may have occurred very early during evolution (McRory & Sherwood 1997). In fact, the organization of the GHRHII/PACAP1 gene of the sea squirt exhibits much similarity with that of the mammalian GHRH precursor gene, while the structure of the GHRH/PACAP2 gene of the sea squirt resembles that of the mammalian PACAP

precursor gene (Fig. 4), suggesting that the two urochordate genes may be orthologous to the two mammalian genes. In that case, the existence of two GHRH/PACAP precursors should also occur in submammalian vertebrate species. Indeed, Southern blot analysis of salmon genomic DNA has revealed the existence of at least two bands hybridizing with a probe including the GHRH/PACAP coding region (Parker *et al.* 1993) and two variants of GHRH/PACAP cDNAs have recently been cloned in the goldfish (Leung *et al.* 1999), confirming the existence of more than one GHRH/PACAP gene in teleosts.

EVOLUTION OF THE DISTRIBUTION OF GHRH AND PACAP NEURONS IN THE BRAIN

In mammals, GHRH-containing neurons are strictly located in the arcuate and the dorsomedial nuclei of the hypothalamus (Mayo *et al.* 1995), whereas PACAP-containing perikarya are widely distributed, not only in the hypothalamus, but also in the thalamus, septum, cortex, amygdala, hippocampal area, cerebellum and pons (Arimura *et al.* 1991, Kovacs *et al.* 1991, 1994, Ghatei *et al.* 1993, Legradi *et al.* 1994, Nielsen *et al.* 1998) (Fig. 5a).

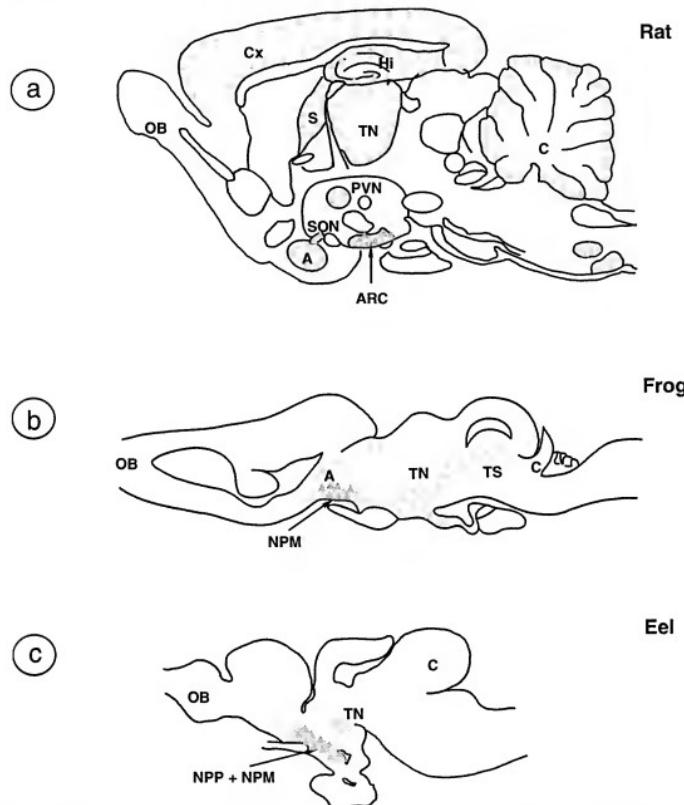


FIGURE 5. Schematic parasagittal sections through (a) rat, (b) frog and (c) eel brain depicting the distribution of GHRH and PACAP perikarya. Red triangles, GHRH-immunoreactive cell bodies; green triangles, PACAP-immunoreactive cell bodies. A, amygdala; ARC, arcuate nucleus; C, cerebellum; Cx, cortex; Hi, hippocampus; NPM, magnocellular preoptic nucleus; NPP, posterior preoptic nucleus; OB, olfactory bulb; PVN, paraventricular nucleus; S, septum; SON, supraoptic nucleus; TN, thalamic nucleus; TS, torus semicircularis. Adapted from Gonzalez *et al.* 1998, Montero *et al.* 1998 and Yon *et al.* 1992.

In birds, the distribution of GHRH neurons has never been reported while the location of PACAP-containing neurons has been studied in the chicken forebrain (Peeters *et al.* 1998a). PACAP-

immunoreactive cell bodies are particularly abundant in the ventral aspect of the supraoptic nucleus, as well as in the magnocellular subdivisions of the preoptic and paraventricular nuclei (Peeters *et al.* 1998a).

In amphibians, unfortunately, the distribution of GHRH neurons has been exclusively investigated in the hypothalamo-hypophyseal system, so that the presence of GHRH-immunoreactive cell bodies has only been described in the magnocellular part of the preoptic nucleus (Marivoet *et al.* 1988). The existence of PACAP-positive perikarya has been reported in the frog hypothalamus as well as in extrahypothalamic areas including the thalamus, amygdala and torus semicircularis (Yon *et al.* 1992) (Fig. 5b). Since, in frog, a GHRH-like peptide and PACAP are encoded by the same gene (Alexandre *et al.* 2000), it is conceivable that GIIRH-like immunoreactivity could have a more widespread distribution than initially described (Marivoet *et al.* 1988). Thus, the location of GHRH-containing neurons in the amphibian brain outside the hypothalamic region deserves to be investigated.

In fish, GHRH-immunoreactive neurons are primarily located in the magnocellular and parvocellular portions of the preoptic nucleus; a few GIIRH-positive neurons are also found in the rostral part of the nucleus lateralis tuberis (Marivoet *et al.* 1988, Olivereau *et al.* 1990). Similarly, PACAP-positive cell bodies are found in the preoptic nucleus and in the nucleus lateralis tuberis (Matsuda *et al.* 1997b, Montero *et al.* 1998, Yoshida *et al.* 1999) (Fig. 5c). The expression of PACAP mRNA in the preoptic area has recently been confirmed by *in situ* hybridization (Satoh *et al.* 1999). The co-localization of GHRH and PACAP in the same regions of the fish diencephalon is consistent with the occurrence of a GHRH-like peptide and PACAP within the same precursor (Parker *et al.* 1993, McRory *et al.* 1995). Interestingly, in teleost fish, fibers immunoreactive for GIIRH and PACAP directly innervate the pars distalis (Marivoet *et al.* 1988, Olivereau *et al.* 1990, Matsuda *et al.* 1997b, Montero *et al.* 1998), suggesting that both GHRH and PACAP may be involved in the control of pituitary hormone secretion.

EVOLUTION OF THE REGULATION OF GH SECRETION BY GHRH AND PACAP

In mammals, GH secretion is primarily under the stimulatory control of GHRH and the inhibitory influence of somatostatin (Bertherat *et al.* 1995). Studies aimed at investigating the effect of PACAP on GII secretion have led to controversial results. Specifically, some data suggest that PACAP stimulates GH release in rat (Goth *et al.* 1992, Hart *et al.* 1992, Jarry *et al.* 1992, Leonhardt *et al.* 1992,

TABLE 1. Relative implication of GHRH and PACAP in the control of GH secretion in vertebrates

TAXA	GHRH	PACAP
Mammals	+++	+/-
Birds	+++	+
Reptiles	?	?
Amphibians	+++	+++
Telosts	+/-	+++

Nagy *et al.* 1993, Wei *et al.* 1993, Velkeniers *et al.* 1994), sheep (Sawangjaroen *et al.* 1997) and cattle (Ishizume *et al.* 1994), whereas other reports indicate that PACAP has no effect on GH secretion (Miyauchi *et al.* 1989, Culler & Paschall 1991, Jarry *et al.* 1992, Sawangjaroen & Curlewis 1994). In humans, intravenous administration of PACAP does not modify plasma GII levels (Chiodera *et al.* 1996) and PACAP is less potent than GIIRH in stimulating GII release from somatotropin adenoma cells in primary culture (Adams *et al.* 1994). In birds, PACAP has been found to induce a dose-dependent stimulation of cAMP formation by chicken pituitary cells. However, PACAP causes only a modest increase of GH release as compared with the robust stimulatory effect of human GIIRH (Peeters *et al.* 1998b). Taken together, these data indicate that PACAP probably plays a minor role in the control of GH secretion in mammals and birds (Table 1).

In amphibians, frog (f) PACAP stimulates GH secretion in the European green frog *Rana ridibunda* (Martinez-Fuentes *et al.* 1994) and the bullfrog *Rana catesbeiana* (S Kikuyama, unpublished data). Frog PACAP stimulates adenylyl cyclase activity in frog pituitary slices (Yon *et al.* 1993) and increases cytosolic calcium concentration in cultured frog somatotrope cells (Gracia-Navarro *et al.* 1992). At least two types of PACAP receptors, i.e. type I receptors (PACAP-specific receptors) and type II receptors (PACAP-VIP receptors) are expressed in the distal lobe of frog and toad (Alexandre *et al.* 1999, Hu *et al.* 2000). Concurrently, it has been shown that human (h) GHRH stimulates GH secretion from bullfrog pituitary cells, and that the hGHRH-induced GH release is inhibited by somatostatin in a dose-dependent manner (Jeandel *et al.* 1998). In addition, we have recently found that fGHRH-like peptide and fPACAP are equipotent in stimulating GII secretion from cultured frog adenohypophyseal cells (S Kikuyama, unpublished data). These observations suggest that, in amphibians, GHRH and PACAP (in addition to

somatostatin) are involved in the neuroendocrine control of somatotrope cell activity (Table 1).

In fish, GHRH has been reported to exert a modest stimulatory effect in the trout *Oncorhynchus mykiss* (Luo *et al.* 1990, Blaise *et al.* 1995), the carp *Cyprinus carpio* (Vaughan *et al.* 1992) and the tilapia hybrid *Oreochromis niloticus* × *Oreochromis aureus* (Melamed *et al.* 1995) both *in vitro* and *in vivo*. In the salmon *Oncorhynchus nerka*, native GHRH produces only a weak stimulation of GH release from pituitary cells (Parker *et al.* 1997). In a primitive teleost, the European eel *Anguilla anguilla*, hGHRH is totally devoid of an effect on GII secretion (Montero *et al.* 1998). In contrast, both PACAP27 and PACAP38 provoke a robust stimulation of GII release from goldfish *Carassius auratus* (Wong *et al.* 1998) and eel pituitary cells (Montero *et al.* 1998). These observations strongly suggest that, in fish, PACAP rather than GHRH plays a physiological role in the control of GH secretion (Table 1). It should be noticed, however, that in the goldfish both type I PACAP receptor and a GHRH receptor are expressed in the pituitary (Chan *et al.* 1998, Wong *et al.* 1998), indicating that not only PACAP but also GHRH may exert some kind of hypophysiotropic activity. In addition, it has previously been shown that, in birds, amphibians and fish, several regulatory neuropeptides such as gonadotropin-releasing hormone (Marchant *et al.* 1989, Lin *et al.* 1993, Melamed *et al.* 1995), neuropeptide Y (Peng *et al.* 1990), thyrotropin-releasing hormone (Harvey *et al.* 1978, Gracia-Navarro *et al.* 1991, Trudeau *et al.* 1992), cholecystokinin (Himick *et al.* 1993), bombesin (Himick & Peter 1995) and corticotropin-releasing hormone (Rousseau *et al.* 1999) are involved in the control of somatotrope cell activity.

CONCLUSIONS

Since the identification of the sequence of secretin (Mutt *et al.* 1970), the characterization of multiple related peptides has provided insight into the molecular evolution of this large peptide superfamily. In the case of GHRH and PACAP, a series of exon and gene duplication events has led to the formation of a subfamily of neuropeptide precursors which exhibit the same overall organization. During vertebrate evolution, the primary structure of GHRH has markedly diverged while the sequence of PACAP has been strongly preserved. In submammalian vertebrates, GHRH and PACAP are arranged in tandem within the same precursor whereas, in mammals, the two peptides are

generated from distinct precursors, suggesting that gene duplication may have occurred in a common ancestor during the transition period between birds and mammals (Fig. 4). Alternatively, submammalian vertebrates may possess two related GHRH/PACAP-encoding genes, one of which would remain to be characterized.

In all vertebrate species studied so far, the secretion of GII is under the dual control of inhibitory and stimulatory neuropeptides. While somatostatin acts as an inhibitor of GH release throughout the vertebrate phylum, the respective roles of GHRH and PACAP as stimulatory factors of somatotrope cells have been radically inverted during evolution (Table 1). Recently, a novel hypothalamic neuropeptide, termed ghrelin, has been characterized for its ability to stimulate GH secretion in mammals (Kojima *et al.* 1999). Identification of this novel neuropeptide in representative submammalian species and investigation of its possible GII-releasing activity will be necessary to understand the phylogenetic history of the control of GII secretion during vertebrate evolution.

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